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MITOGENIC ACTIVITY IN OVINE UTERINE FLUIDS: CHARACTERIZATION OF A GROWTH FACTOR ACTIVITY WHICH SPECIFICALLY STIMULATES MYOBLAST PROLIFERATION[$]

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Fluids produced by the uterus of pregnant sheep (OUF-ovine uterine fluids) were assayed for mitogenic activity in a thymidine incorporation assay. A dose-dependent mitogenic activity was observed in OUF which exceeded that of adult ovine plasma or fetal bovine serum. Uterine fluids were capable of stimulating thymidine incorporation in mouse 3T3 fibroblasts, rat L6 myoblasts, ovine trophoblast-derived cells, HeLa S3 cells, and bovine aortic endothelial cells. The greatest stimulation was observed in L6 myoblasts. The name ovine uterine-derived growth factor (ovine UDGF) has been suggested for this activity.

Regulation of proliferative activity in eukaryotic cells is dependent upon the actions of peptide growth factors. Growth factors have generated interest not only because of their role in normal growth (1) but also due to their implication in the development of the transformed phenotype (2,3). The discovery of shared homology between the epidermal growth factor receptor and the oncogene erb-B (4,5) as well as between platelet-derived growth factor and the sis oncogene (6,7) both support this contention.

Until recently little attention has been paid to the mitogenic activities present in fluids produced by maternal and fetal tissues during mammalian development. Although specific proteins have been identified in fluids from maternal (8-10) and embryonic tissues (9,13), little is known concerning the components which stimulate proliferation of fetal cells. The rapid

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Abbreviations Used: HBS, Hepes-buffered saline, alpha-MEM, alpha modification of Eagle's minimum essential medium, OUF, ovine uterine fluid(s), FBS, fetal bovine serum, GFU, growth factor unit(s), TES, Tris-EDTA-SDS solution, FGF, fibroblast growth factor, SD, standard deviation.

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proliferation of all tissues during early development suggests that fluids which emanate from the uterine lining and are absorbed by the fetus (9,14) are a likely source of growth factors.

The surgical technique of unilateral ligation of a single uterine horn of ewes allows collection of large volumes of concentrated uterine fluids produced by the endometrium (9,15). These fluids are normally absorbed by the fetal tissues and do not accumulate. Since the maternal side is surgically isolated by the ligature, the fluids which collect and any growth factors residing therein, must be of maternal origin. The large volume and high concentration of this material offer distinct advantages over the dilute products recovered by uterine flushing (15). We have characterized the mitogenic activity(s) found in ovine uterine fluids collected from unilaterally ligated uterine horns at a late stage of pregnancy.

MATERIALS AND METHODS

Cell Lines and Culture - The myoblast cell line, clone L6-5 derived from rat skeletal muscle, was obtained from C.P. Stanners (McGill University)(17) and was originally isolated by D. Yaffe (18). The HeLa S3 cell line of human epithelial carcinoma-derived cells and the mouse 3T3 fibroblasts were obtained from B.H. Sells (University of Guelph) (19). These three cell lines were grown in 25 cm^2 tissue culture flasks or 24-well plates (Corning) in alpha-MEM (Flow Laboratories) supplemented with 10% (v/v) fetal bovine serum (Flow) and the cultures were incubated at 37°C, 100% humidity and 5% CO_2 in air. All cell lines were passaged prior to attaining confluence by trypsinization and diluted 1:3 - 1:5 in fresh growth medium.

Primary cultures of aortic endothelial cells were isolated from bovine aortas by a modification of published procedures (20). Pieces of fresh descending thoracic aorta (approximately 5x5 cm) were obtained at slaughter, and the lumenal surfaces were rinsed 4 times in sterile HBS (10 mM HEPES, pH 7.2, 0.9% NaCl). The lumenal surface was digested with 1 mg/ml collagenase (Worthington, CLSII) in HBS containing 0.15 mg/ml $CaCl_2$ and 0.1 mg/ml $MgCl_2$ at 37°C for 30 min. The endothelial cells were gently scraped from the lumenal surface with a sterile Teflon spatula and rinsed in alpha-MEM containing 20% FBS. Cell suspensions were gently centrifuged for 2 min in a clinical centrifuge and resuspended in alpha-MEM with 20% FBS prior to plating in 25 cm^2 tissue culture flasks coated with sterile 0.1% (w/v) gelatin. Bovine aortic endothelial cells were transferred from coated flasks to uncoated flasks by passage 5. Cell line BA53-90d, used in this study, was derived from a 90 day bovine fetus. The cells were uniformly endothelial in morphology (cobblestone appearance at confluence) and were used between passages 25-35.

The OE-1 cell line was established from a preimplantation ovine embryo as previously described (21). Briefly, a single day 11 ovine embryo was cultured in HEPES-buffered Ham's F-10 medium (Gibco) supplemented with 10% (v/v) FBS, 110 ug/ml sodium pyruvate, and 100 ng/ml epidermal growth factor (Sigma). After 13 days in culture the embryo had attached and an explant growth originating from the trophectoderm covered an area approximately 0.2 cm^2 in the culture well. The explant was dispersed by trypsinization, and cells which grew as a monolayer were expanded through serial passage at dilutions of approximately 1:3. Sufficient numbers of cells were available for study by the 4th passage (25 cm^2 flasks). Cells used in this study were at the 6th to 13th passage.

Collection of Ovine Uterine Fluids - Uterine fluids were collected from the nonpregnant horn of ewes on gestational day 120 as previously described (15). The fluids were immediately divided into aliquots and stored at -70°C. Before use, the aliquotes were thawed, their protein concentration determined (22),

and they were diluted to 2.5 mg/ml total protein with HBS. Suspended particles were removed by centrifugation (14,000Xg, 2 min, 4°C), and the supernatant was sterilized by filtration through a 0.2 um filter (Schleicher & Schuell).

Growth Factor Assay - Growth of the cell lines was determined by the rate of ^{3}H-thymidine incorporation into acid precipitable material as previously described (23) with some modification (17). Each cell line was propagated in the appropriate growth medium and plated at approximately 50% confluence on 24-well tissue culture plates (Corning). The cells were then allowed to become quiescent in one of two ways: (1) allowed to grow to confluence and maintained without a change of medium for 5 days or (2) allowed to grow to confluence and the medium changed to alpha-MEM without serum supplement for 24 hr. At this time the medium was reduced to 150 ul conditioned medium per well, and the growth factor activity assay was conducted. A total of 100 ul of growth supplement and HBS was added to each well. Each plate included a titration of each test OUF (diluted to 2.5 mg/ml and sterilized by filtration) up to 20% (v/v), several negative control wells in which 100 ul of HBS was added, and a positive control titration of up to 20% (v/v) FBS. To each well was added 10 ul of HBS containing 2.5 uCi of ^{3}H-thymidine (10 uCi/ml final, New England Nuclear). The contents of the plates were mixed and incubated for approximately 24 hr at 37°C. The assays were stopped by removing the labeled medium, rinsing out each well with ice-cold HBS (400 ul/well) and lysing the cells with 80 ul/well of TES (10 mM Tris-HCl, pH 7.6, 1 mM Na_2EDTA, 1% w/v sodium dodecyl sulfate). Acid precipitable counts were determined for each lysate as previously described (17). The contents of each well were pipetted onto a Whatman 540 paper disc and allowed to dry. Discs were then batch precipitated and washed at room temperature, sequentially, in 300 ml/50 filters for 20 min, in 20%(w/v) and then 10%(w/v) trichloroacetic acid, absolute ethanol, diethyl-ether, and absolute ethanol and allowed to dry. The filters (including several blanks) were then counted in an LKB liquid scintillation counter in 5 ml of toluene-omnifluor (New England Nuclear). Each titration point was calculated as the mean of the CPMs from 2-4 wells less the mean CPMs obtained with the blank filters. The level of stimulation obtained in the 50 ul point in the FBS titration above the level observed for the HBS negative controls in each individual plate was defined as 2 GFU: ie. FBS was defined as containing 2 GFU in each assay as previously described (23).

Growth Factor Characterization - OUF samples were subjected to ultrafiltration (Amicon centricon microconcentrator) to retain native protein complexes of >30,000 daltons as per manufacturers specifications. OUF samples, diluted to 2.5 mg/ml and centrifuged 5 min at 10,000xg (4°C), were subsequently fractionated by centrifugation through the microconcentrator for up to 2 hr at 5,000xg (4°C) to collect the filtrate of less than 30,000 daltons. HBS was then added to the inverted device, and the whole assembly was recentrifuged to collect the greater than 30,000 dalton fraction off the filter at approximately the original concentration. Both fractions were tested to determine which contained the mitogenic activity.

RESULTS AND DISCUSSION

The amount of mitogenic activity in each OUF sample was determined by titration of the OUF in a growth factor assay based upon the incorporation of ^{3}H-thymidine into quiescent 3T3 cells (Fig.1). On each plate tested the diluted OUF were compared to a test titration of FBS (23). One growth factor unit was defined arbitrarily as 50% of the response of quiescent 3T3 cells to 50 ul of FBS (20% v/v) per 250 ul total of conditioned medium and supplementary HBS. In each case the response was found to be dose dependent.

Six different OUF and plasma from adult sheep were tested for growth factor activity in the thymidine incorporation assay (Table I). The mean GFU in OUF

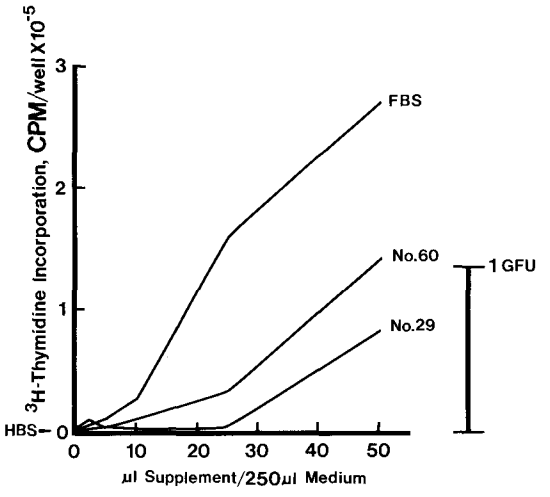

Figure 1: A representative growth factor activity assay including two independent OUF samples as well as both positive and negative controls. A serial titration of OUF samples No.29 and No.60 were tested against a titration of FBS positive and HBS negative controls for their ability to stimulate incorporation of thymidine over 24 hr in quiescent 3T3 cells. Calculation of growth factor unit (GFU) activity for this assay is shown. These two OUF samples were diluted 3.4X (No.29) and 3.1X (No.60) prior to testing, to bring them to a uniform 2.5 mg/ml total protein. SD for each time ranged between $1.5x10^2$ and $6.08x10^4$ cpm or an average SD of 22% of the mean at each time point.

ranged from 3.2-16.0 GFU/mg protein compared to 1.54 GFU/mg for FBS. In all cases the GFU in OUF were much higher than those found in plasma from adult sheep (0.88 GFU/mg protein) ranging between 3.6 and 18.2 times higher. This suggests that even though OUF contain detectable levels of plasma proteins by electrophoretic analysis (8,24), plasma proteins do not seem to be the source of the growth factor activity observed. Because plasma did contain low GFU activity it is possible that some of the observed activity occurred because serum transferrin or other serum-derived growth factors, while being

TABLE 1

GROWTH FACTOR ACTIVITY IN OVINE UTERINE FLUIDS

OUF	PROTEIN CONC. (mg/ml)	VOLUME RECOVERED (ml)	GROWTH FACTOR ACTIVITY CONC. (GFU/ml)	TOTAL (GFUx10^{-3})	SPECIFIC ACTIVITY (GFU/mg +/-SD)[a]	
A	8.4	653	134	87.8	16.0 +/-2.0	(5)
B	7.7	273	105	28.6	13.6 +/-1.6	(5)
C	26.6	677	98	66.3	3.68 +/- 0	(2)
D	73.2	8	252	2.0	3.44 +/-0.05	(3)
E	39.1	ND	428	ND	10.96 +/-0.53	(2)
F	27.3	ND	87	ND	3.2	(1)
PLASMA	64.4	NA	57	NA	0.88	(1)
FBS	26.0	NA	40[b]	NA	1.54	

[a] Values are the mean and SD of n determinations, where n is given in parentheses.

[b] The GFU/ml for FBS is calculated from the arbitrarily defined value of 2.0 GFU/50 ul FBS therefore no SD is given.

ND - not determined, NA - not applicable, A - OUF No.29, B - OUF No.60.

transported by the uterine lining, undergo significant concentration (25). It seems more likely, however, that most of the observed activity emanates from the uterine lining which has been shown to synthesize a variety of secreted proteins in culture (8).

By definition, FBS contains 1.54 GFU/mg protein in this assay (Table I). When the dilution factor for each OUF is taken into account, the net mean GFU activity for OUF ranged from 3.2-16.0 GFU/mg protein. This is 2.1-10.4 times higher than that observed in FBS per mg protein. OUF protein concentration was not correlated with GFU activity in each sample (data not shown). The bulk of the protein present in each OUF sample was unrelated to the level of GFU activity observed.

Diluted samples of OUF were moderately stable during heat treatment (Table II). Treatment at 60°C for 20 min resulted in a 10%-22% loss of growth factor activity. Treatment at 100°C for 20 min, however, resulted in an 18%-100% loss of activity suggesting a progressive loss of GFU activity. GFU activity was also able to withstand at least 3 cycles of freezing and thawing without being diminished. Preliminary evidence suggests that the GFU activity is not basic-FGF because it has little or no affinity for heparin-agarose in 0.15 M NaCl, 10 mM KH_2PO_4, pH 7.4 (data not shown) (26).

An estimation of the approximate molecular weight limit of the GFU activity in OUF was obtained by ultrafiltration on a 30,000 dalton exclusion filtration device. In three separate experiments from 51.0-99.7% of the growth factor activity was retained by the exclusion filter indicating a size of greater than 30,000 daltons for the native form. The presence of some activity in the fraction which passed through the filter may reflect the presence of other smaller growth factor activities in OUF.

To determine whether OUF possessed any tissue specificity, the thymidine incorporation assay was modified to accommodate a variety of cell lines representing five different cell types (Fig.2). Reducing the period of

TABLE II

HEAT STABILITY OF GFU ACTIVITY

OUF No.	% OF INITIAL GFUs AFTER 20 MIN AT 60°C	% OF INITIAL GFUs AFTER 20 MIN AT 100°C
A	78	ND
B	90	50
C	ND	0
D	96	51
E	ND	35
F	ND	82
FBS	ND	30

Samples of each OUF were diluted and filter sterilized and heat treated immediately prior to assaying for growth factor activity. GFU activity was evaluated after treatment at 60°C or 100°C for 20 min. Duplicate native samples were held on ice during treatment for comparison. ND - not determined.

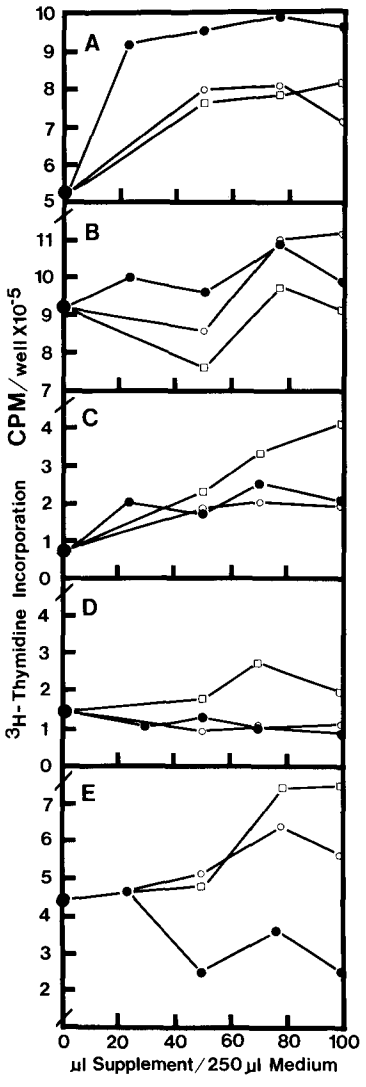

Figure 2: OUF growth factor activity assays on five different cell types. The mitogenic activity of OUF no.29 (open circles) and no.60 (open squares) were compared to that of FBS (small closed circles) in cells which had been grown to confluence and changed to serum-free alpha-MEM for 24 hr. Unstimulated cultures received an equivalent volume of HBS (large closed circles). A) L6-5 rat skeletal muscle myoblasts (SD range $1.9x10^3$ to $3.33x10^4$ cpm or 11.6% of the mean on average), B) HeLa S3 epidermal carcinoma-derived cells (SD range $3.35x10^4$ to $22.4x10^4$ cpm or 9.7% of the mean on average), C) OE-1 ovine trophoblast-derived cells (SD range $8.0x10^2$ to $36.7x10^4$ cpm or 9.3% of the mean on average), D) BA53-90d bovine endothelial cells (SD range $2.7x10^3$ to $9.7x10^4$ cpm or 17.3% of the mean on average), E) 3T3 mouse fibroblast cell line (SD range $6.6x10^2$ to $27.8x10^4$ cpm or 18.4% of the mean on average). Note that 3T3 cells were not made fully quiescent in this modified assay.

quiescence from 5 to 1 day increased the residual incorporation observed in HBS treated controls but was necessary because several of the cell lines did not proliferate readily after extended periods of quiescence.

A differential effect of OUF stimulation was observed in different cell types. Mouse 3T3 fibroblasts were not fully quiescent under this modified

treatment and showed only a slight stimulation in thymidine incorporation over FBS. Higher FBS concentrations proved somewhat inhibitory to nonquiescent 3T3 cells. Incorporation was only marginally increased by the presence of FBS over HBS during the next 24 hr. Incorporation by bovine aortic endothelial cells was unaffected by greater amounts of FBS and was only slightly enhanced by one of the OUF samples. Ovine trophoblast-derived cells were moderately stimulated by increased FBS or OUF concentration. HeLa S3 epidermal carcinoma-derived cells were also stimulated by increasing concentrations of FBS and by OUF at higher concentrations. Rat L6-5 skeletal myoblast cells were very sensitive to both FBS and OUF concentration. Myoblasts consistently responded to increasing concentrations of OUF with increased incorporation of thymidine.

The effect of OUF upon skeletal myoblasts was striking compared to its effect upon all other cell types tested. Activity was 1.6 GFU (or 81% of FBS at 50ul) after a 3.1 or 3.4-fold dilution (11.8 or 14.1 GFU/mg protein for OUF compared to 7.2 for FBS in L6 cells). Also, in myoblasts and trophoblasts the effect of OUF on ^{3}H-thymidine incorporation had not come to a plateau. The data suggest that OUF contain a growth factor-like activity which has a marked and specific effect upon trophoblast and myoblast cells in culture. The trophoblast is the first cell type to differentiate in the ovine embryo so that this effect was not unexpected. However, the effect of OUFs upon myoblasts was surprising because few muscle specific growth factors have been reported (25,27).

Due to its unusual characteristics with regard to cell type specificity it is possible that OUF contain a novel growth factor. If so, we suggest the name ovine UDGF: ovine uterine-derived growth factor for this activity (26,27).

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